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Continuous culture of sulphur-oxidizing microorganisms for metal recovery

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Abstract

Nowadays heavy metal contamination has become a global problem. So, it is necessary to find a way to remove heavy metals and biological extraction can be a good alternative from physical or chemical. Biological extraction ahs low energy demand and it is considered a green and environment friendlier technique. The most commonly biological method used in metal recovery is called bioleaching. An example of bioleaching could be use sulphur oxidizing bacteria (SOB) to oxidized elemental sulphur to sulphuric acid, lowering pH and improving heavy metals solubilization. The present work aims to study copper recovery and to enrich sulphur oxidizing microorganisms in a reactor to obtain an effluent to be able to carry out the bioleaching process. A continuous stirred tank reactor with mixed of *Acidithiobacillus thiooxidans* and sulphur-oxidizing microorganisms to achieved the effluent, had been tested. Also, a bioleaching Jar-Test in order to assess copper solubilization as a function of sulphuric acid dose, total solids content and stirring time. After activating the microorganisms, the reactor was able to start up properly, achieving an effluent with a 0.06 M of sulphuric acid concentration. In addition, Jar-Test was reached a copper solubilization between 148.25 mg Cu/kg sludge and 203.86 mg Cu/kg sludge. The factor combination that obtained the best copper solubilization was: 2% total solids content, 6 days of stirring time and 3 g S°/L transformed in sulphuric acid dose.

Keywords: sulphur-oxidizing microorganisms, Jar-Test, copper solubilization, continuous stirred tank reactor, heavy metals, bioleaching.

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1. Introduction

1.1. Compost from wastewater treatment sludge

Every year, around to 8, 4 and 2 billion tonnes of waste activated sludge (WAS) is produced in the US, China and Europe because of wastewater treatment (2009), but this number has an increasing tendency in the near future (Meulepas et al. 2015). There is the opportunity to use municipal sewage sludges and wastes, once digested or composted, to improve the properties of impoverished soils. In addition, the EU legislation considers that this kind of compost can have substantial benefits for climate change and its recycling is economically sustainable since it reduces the usage of chemical fertilizers (Carbonell et al. 2011). Nevertheless, some problems must be addressed, as “an above-EU limits” of heavy metals (HM) content (Meulepas et al. 2015). There are three groups of HMs that deserve more attention (Ballester et al. 2017): (1) HMs with a considerable environmental risk because of their toxic effect (Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn); (2) HMs with high value, even after their recovery from wastes (Pd, Pt, Ag, Au, Ru); and (3) Radioactive metals (U, Th, Ra, Am). In this work, the focus will be on those of the first group.

1.2. Heavy metals and its problematic

Nowadays HM contamination has become a global problem. The main causes are the increase of the urbanized areas, changes in lands´ use and industrialization, especially in areas with high population density and in developed countries (Afonne and Ifediba 2020). HMs can be found naturally in the environment, soils and food, as well as they are extensively used in farming, agriculture and manufacturing processes. HMs are transferred to the environment because of uncontrolled waste or wastewaters discharges, but also under controlled waste or wastewater management strategies. One example would be the management of municipal solid wastes (MSW) through composting: there are many sources of HMs in the compost, especially derived from municipal solid waste (Smith 2009).

The main problems are caused by HMs accumulated in soils (Table 1) (Smith 2009). Also, the HMs accumulation in the bone matrix and fatty tissues of human bodies entails some health problems, as an essential nutrients depletion, a central nervous system deficit, growth retardation or diseases of the cardiovascular, gastrointestinal, haematological, hepatic, renal, neurological, reproductive and immune systems (Afonne and Ifediba 2020). In this regard, the riskiest HMs are Zn, Cu, Ni, Cd, Pb, Cr and Hg. These HMs can be found in composts from MSW or WAS in higher amounts than in soils (Carbonell et al. 2011). Moreover, the concentration of HMs increases during the composting process, as there is a concentration effect because of an organic matter and volatile solids during the microbial degradation (Smith 2009). Due to this reason, the EU agreed a maximum allowable number of HMs in fertilizers or soil conditioners. In Spain, currently, these values (Table 2) can be found in the *RD 506/2013*[1] where fertilizer products are classified into three categories (A, B, C) depending on HMs content. The A category is the most restrictive and therefore the one with the best quality.

Table 1 Environmental issues that can happen when HMs accumulate in the soil.

HMs	Environmental issues
Zn, Cu, Ni	Reduced plant growth (there is no evidence that Cr from the compost cause damage to the plant)
Cd	Introduction to the human food chain through crops
Cd, Pb, Hg	Introduction to the human food chain through the soil (in infants)
Cd, Pb	Introduction to the human food chain through cattle intake
Cu, Pb	Impact on animal health
Zn	Impact in the processes of the soil microorganisms

Table 2 Maximum values of heavy metals for fertilizer products according to the current Spain legislation.

HM	Concentration limits Solids: mg/kg of dry matter		
	Liquids: mg/kg		
	A Category	B Category	C Category
Cadmium	0,7	2	3
Copper	70	300	400
Nickel	25	90	100
Lead	45	150	200
Zinc	200	500	1.000
Mercury	0,4	1,5	2,5
Chrome (total)	70	250	300
Chrome (VI)	Not detectable by official methods		

[1] *Real Decreto 506/2013, de 28 de junio, sobre productos fertilizantes.*

1.3. Heavy metals recovery

The removal of HMs from the environment is a good alternative to emissions control and to prevent their accumulation. Physical or chemical technologies are used, but these have limitations in practical applications (Chen and Lin 2004). For example, chemical extraction can be done with some leaching agents (sulphuric acid, iron hydrochloride, ammonium hydrochloride, iron sulphate, ammonium sulphate, hydrochloric acid and nitric acids) that can achieve an efficient and quick solubilization of metals (99%). The main disadvantage of using chemical extraction is the high cost of chemical reagents and energy requirements (Potysz et al. 2016). On the other hand, physical methods, such as membrane filtration, have some advantages like the removal of suspended solids and organic compounds together with HMs, or their selectivity. Nevertheless, the disadvantage is a low flow rate and high cost (Raouf MS and Raheim ARM 2016).

Biological extraction can be a good alternative because of (i) its low energy demand (temperature and pressure needs are much lower); (ii) it is considered a green and environment friendlier technique (lower waste generation and smaller CO_2 footprint). On the other hand, the disadvantages are (a) longer time to get a HMs extraction economically profitable; (b) it requires a strict monitoring of the process parameters (Johnson 2014); and (c) furthermore, a biological process used to have a slow kinetics, not available for commercial scale (Naseri 2019). Thus, because of the legislative constraints, it’s essential to find environmental friendlier and economically reliable routes.

1.4. Bioleaching

The most commonly biological method used in metal recovery is called bioleaching. There are two different mechanisms:

- Direct bioleaching. The bacterium catalyses the oxidation of insoluble metal sulphites to soluble metal sulphates.
- Indirect bioleaching. Elemental sulphur or reduced sulphuric compounds are oxidized to sulphuric acid by these bacteria, lowering the pH and improving metals solubilization (Chen and Lin 2004). Metals in form of sulphites and oxides are solubilized in metal cations.

Then the next step is the purification of solubilized metals in the leachates by processes such as solvent extraction, adsorption, ion exchange, membrane separation, selective precipitation, etc. (Srichandan et al. 2019). During the indirect bioleaching, the maximum solubilization efficiency of metals corresponds to optimum growth conditions for bacteria. Then the effectiveness of these bacteria is determined by their ability to lower the pH through the production of sulphuric acid because acidic conditions are favourable for the metal extraction, through the replacement of metals by protons (Potysz et al. 2016).

It should be considered that when elemental sulphur is used as a substrate, it becomes the limiting parameter of the process since generally acid production, and so pH changes, are affected by the sulphur addition. However, if an excess of sulphur is added, the residual sulphur will cause reacidification of the compost (Chen and Lin 2004).

1.4.1. Microorganisms

Microorganisms with the ability to do the bioleaching process can be classified into 3 different groups (Baniasadi et al. 2019): (i) Chemolithoautotrophic bacteria that carry out acidolysis[2] and redoxolysis[3] mechanisms, such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. (ii) Organic acid producing fungi that carry out acidolysis and complexolysis[4] mechanisms, such as *Aspergillus niger, Penicillium simplicissimum.* (iii) Cyanogenic bacteria that carry out complexolysis, such as *Chromobacterium violaceum*, *Bacillus megaterium*.

The first recognized operation, in metal recovery using a biological technique, was 20 years after of the first bacterium was discovered (Johnson 2014). The same biological process has been used, unknowingly, to extract metals from mines in Spain, United Kingdom and China for centuries. This “new” biotechnology was “discovered” in the 1960s by the Kennecott Copper Corporation by wanting to extract copper from waste rocks at the Bingham Canyon in Utah and at the Chino mine in New Mexico (Johnson 2014).

Chemolithoautotrophic bacteria such as *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are the most used in bioleaching, thanks to their ability to tolerate high concentrations of HMs and to grow up under extreme conditions of acidic pH (Panda et al. 2018). The ability to leach heterotrophic metal such as *Pseudomonas*

[2] Acidolysis: the oxygen atoms that cover metal components are protonated.
[3] Redoxolysis: is the solubilization of metal through oxidation-reduction reactions. Under anaerobic conditions hydrogen or sulphur work as electron donors.
[4] Complexolysis: mechanism of fungi and cyanogenic bacteria for metal recovery.

has also been studied. The problem is that these bacteria need neutral pH in order to grow up, causing a lower solubility of metals as acidic conditions are necessary (Potysz et al. 2016).

Some sulphur oxidizing bacteria (SOB) are photoautotrophic, obtaining energy from light; others are chemolithotropic, obtaining energy by oxidizing sulphur or sulphur compounds and using oxygen as an electron acceptor (aerobic) or nitrates/nitrites (anaerobic). It is considered that chemolithotropic SOBs have better results regarding sulphur removal. Chemolithotropic SOBs are a Gram-negative bacteria group, known as *colourless SOBs*. They acquire their energy via oxidizing inorganic sulphur compounds (sulphide, sulphur and thiosulphate) and consume CO_2 as carbon source (Pokorna et al. 2015). Their optimum temperature and pH ranges are 4°C to 90°C and 1 to 9, respectively, most of them thriving best in mesophilic (30-35°C) or thermophilic (55-57°C) conditions (Tang et al., 2009).

1.4.2. Sulphur cycle

Sulphur can be found in different states and the most significant are: -2 (sulphites and reduced organic sulphur), 0 (elemental sulphur) and +6 (sulphate). Chemical and biological agents are responsible for transforming sulphur into different states. The biogeochemical cycle that explain these transformations is composed by oxidation/reduction reactions. Then H_2S (reduced form of sulphur), can be oxidized to sulphur or sulphate by a wide assortment of microorganisms (Figure 1). At the same time, sulphate may again be reduced to sulphite by the SOBs. Some SOBs and other specialized species can carry out sulphur deprotonation. This is an energy generation process where elemental sulphur or thiosulphate as an electron donor and acceptor, resulting in the simultaneous formation of sulphate and sulphite (Tang et al., 2009).

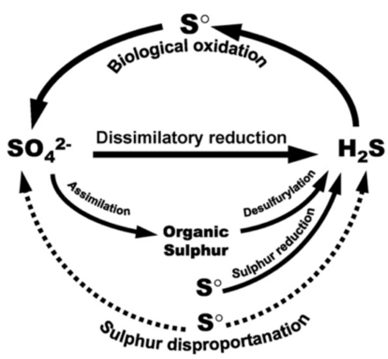

Figure 1 Sulphur cycle of the Sulphur Oxidizing Bacteria

2. Objectives

Sulphur-oxidizing bacteria can solubilize heavy metals ions adsorbed or linked to organic matter by bioleaching. This work pretends to study the integration of the continuous culture of SOBs and the bioleaching process for copper recovery at bench-scale. The main performance parameters, as the copper ion solubilization index, of the whole process will be monitored based on physic-chemical characterization of influents and effluents of the integrated system.

3. Materials and methods

3.1. SOB reactor operation

The SOB reactor operation comprised in three steps: (i) re-activation and production of the inoculum; (ii) inoculation and operation in fed-batch mode; and (iii) continuous operation.

3.1.1. Culture

The microorganisms introduced into the reactor have two different sources. The first source (S1) is an *Acidithiobacillus thiooxidans* pure culture number 14887, obtained from DSM (Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulcuten GmbG, Germany). The culture was maintained at 4 °C until its activation. The second source (S2) is itself a mixture of a pure *Acidithiobacillus thiooxidans* culture and a non-pure culture produced in previous works (Tobias, 2018; Llorens, 2017). The non-pure culture was initially sampled in an MSW treatment plant (Centre Comarcal de Tractament de Residus del Vallès Oriental, Granollers) and then enriched in SOB microorganisms. Although, there was no characterization of the used culture, the presence of SOBs was confirmed because of the pH decreasing with the sulphur conversion during its activation step.

The medium used for the re-activation was like previous works Tobias, (2018) and Llorens, (2017). Micro and macronutrients concentrations are shown in Table 3. The medium (20 L) were prepared with distilled water and maintained at 4 °C until its used.

Table 3 SOB medium nutrients concentrations.

Nutrient	Concentration (g/L)
$(NH_4)_2SO_4$	0.200
$CaCl_2 \cdot 2H_2O$	0.250
$FeSO_4 \cdot 7H_2O$	0.009
$MgSO_4 \cdot 7H_2O$	0.500
KH_2PO_4	3.000
S^0	10.000

3.1.2. SOBs activation and reactor inoculation

The S2 was stored at ambient temperature in 17 vials (Figure 2), 2 flasks as control (one positive and another negative) and 15 flasks with SOB media. Those cultures with the lowest elemental sulphur content (the sulphur was easily visible due to its yellow colour) were chosen as inoculum (7 flasks). In addition, a 1 sample was taken as a new negative control.

Figure 2 Erlenmeyer flasks with culture of sulphur-oxidizing microorganisms obtained in 2018 (source 2)

Then, the selected cultures were cultured in fresh media (Figure 3) to re-activate SOBs. Their growth was detected as a turbidity increment or as a pH decrement. The preparation procedure was:

1. Nutrients for SOBs. A mineral medium with 10 mL of SOB medium were added in 50 mL falcon tubes. It was carried out inside the laminar flow cabinet to make the process sterile.
2. Elemental sulphur addition. 0.1 g of powdered elemental sulphur were introduced into the falcon tubes. Elemental sulphur was crushed with a hammer.
3. Inoculation. 50 µL of the selected S2 were placed on the falcon tubes. Erlenmeyer flasks (Figure 2) could only be opened in a fume hood, because of the amount of acid that it was inside.
4. pH monitoring. Th pH of new cultures were monitored at day 0, 11, 17 and 134 (Table 6).

a) b) c) d) e)

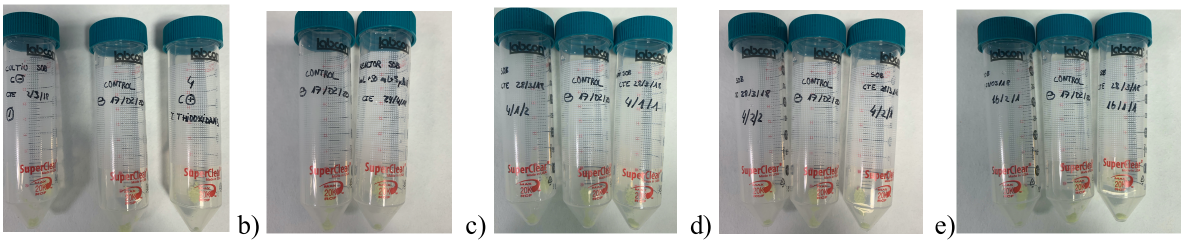

*Figure 3 Images (17/02/20) of the SOBs activation. **a)** C-: negative control made at 02/03/18; C+: positive control made at 02/03/18; M0: negative control made at 17/02/20; **b)** M0: negative control made at 17/02/20; M1: 50 mL of SOB medium + 1 mL of second subculture of mixed inoculum + 0,5 g of S°; **c)** M0: negative control made at 17/02/20; M2: 4/1/1; M3: 3/1/2; **d)** M0: negative control made at 17/02/20; M4: 4/2/1; M5: 4/2/2; **e)** M0: negative control made at 17/02/20; M6: 16/1/1; M7: 16/2/1.*

A second culture was done to increase the inoculum quantity. For that purpose, 1 mL of each corresponding Erlenmeyer's (source S2; Figure 2) were taken and centrifugated at 13.000 rpm for 5 minutes. Then the supernatants were discarded, while the concentrated fractions were diluted at 1/100 with the SOB medium inside 500 mL Pyrex bottles. A final volume of 100 mL was produced.

In parallel, 3 mL of the pure culture of *A. thiooxidans* (source S1) were taken and put also inside a 500 mL Pyrex bottle with the SOB medium. A final volume of 100 mL was produced.

Finally, the reactor was inoculated with 0.6 L of grown up SOBs culture and 0.9 L of SOB medium. After 6 days, a second addition of 1 L of SOB medium was done to fulfil the working volume (2,5 L) of the reactor. Then, the continuous operation of the reactor was started. During all this step, the decrease of pH and the sulphate concentration were monitored to control the performance.

3.1.3. Continuous operation

A continuous stirred tank reactor (CSTR) made of Pyrex glass with a total volume of 3 L (Figure 4) was used for the continuous operation. The influent and effluent flows are stored in the corresponding 1 L bottles, also made of Pyrex glass. A secondary storage of the effluent is done in a 25 L bucket (emptied once a week). The feed bottle is kept stirring using an agitator, to maintained the powdered sulphur homogenised with the medium. A peristaltic pump with two heads is used, one to feed and one to withdrawal the reactor. An overhead mechanical stirrer keeps a homogenised media inside the reactor, using a slow mixing rate. The reactor is aerated with the ambient air, using a small blower; the incoming air is passed through a water trap. The reactor is operated at 28°C. All the experimental set-up is located behind a methacrylate screen that avoid accidental acid splashing.

Figure 4 Continuous stirred tank reactor to be used during the experiment

The influent is composed of elemental sulphur and a similar nutrients media as batch cultures. A concentrated medium is prepared and then diluted (1:20) with deionised water, poured into the glass 1 L bottle to feed the reactor. Also, powdered sulphur (S^0) is added at a concentration of 10 gS^0/L.

Based on previous works (Tobias 2018), the reactor is operated with a hydraulic retention time (HRT) of 16-26 days. Under this value, the expected pH is 1.5-2.5 while the expected sulphate (SO_4^{2-}) content in the effluent is >10 gSO_4^{2-}/L. The SO_4^{2-} concentration is an indicator for S^0 consumption in the reactor. SOBs can produce 1 mol of sulphate per 1 mol of S^0 consumed.

The operation of this reactor includes weekly analytical and maintenance tasks. The control analytics are pH and sulphuric acid concentration. For those purposes, samples of 20-35 ml are taken 2-3 times a week (Monday to Friday). Both pH and sulphuric acid are measured just after taking the sample, the rest was kept at the freezer (-21 °C) until further use. The maintenance tasks of the system included replacing the medium on the feed bottle, controlling the water level on both water trap (incoming air) and water heating system, pipe cleaning and monitoring of working volume of the reactor and effluent & influent daily flows.

3.2. Bioleaching Jar-Test

A jar-test equipment (Figure 5) was used to assess the solubilization degree of HM ions. The procedure is based on Chen et al. (2004) and Liu et al. (2012). The objective of this experiment is to assess the effectiveness of HM solubilization as a function of acidic dose, total solid content, and stirring time. The summary of the factor's combination is shown in Table 4. In this work,

- A mixture of primary and secondary sewage sludge from a municipal wastewater treatment plant was used: the mixed sludge (10 L) was taken from EDAR La Llagosta (Barcelona). It was maintained at 4 °C to avoid any change. This sludge was used diluted with deionised water 2 and 10 times.
- Also, among HMs, copper (Cu) was selected as metal ion model to assess the solubilization.
- Three and six days were chosen for the stirring time, based on mentioned references that concluded these times as the best results.
- The best S^0 doses of S^0 are 1 g S^0/L and 3 g S^0/, based on mentioned references.

Figure 5 Jar-Test equipment

Table 4 Factors combination of the Jar-Test.

		TIME (days)				
		3		6		*Sulphuric acid dose (expressed as g S^0/L)*
Dilutions (total solids content)	**1**	*1*	*3*	*1*	*3*	
	1/2	*1*	*3*	*1*	*3*	
	1/10	*1*	*3*	*1*	*3*	

The experiment is done at room temperature (24-25ºC), following this procedure: (i) samples are introduced in jars, with different total solid content; (ii) different acid doses are introduced into the jars, at the same time; (iii) then samples are shaken (180 rpm) for 3-6 days, measuring pH, conductivity and weightless to monitor water evaporation (1-2 times per day); (iv) the stirring is stopped and samples are settled for 2-3 days; (iv) the concentration of Cu is quantified. 3 replicas were made for each jar-test combination.

Regarding the S^0 doses, elemental sulphur is replaced by sulphuric acid or for the equivalent quantity of SOB reactor´s effluent for the jar-test experiment. According to previous work (Tobias, 2018), the concentration of the sulphuric acid in the effluent of the reactor is at 0.07 M. Thus, the S^0 dose was calculated (eq. 1) into H_2SO_4 dose.

Eq. 1 $\frac{\mathbf{1\ or\ 3}\ g\ S}{L\ material} \times \frac{\mathbf{1}\ mol\ S}{\mathbf{32}\ g\ S} \times \frac{\mathbf{1}\ mol\ H_2SO_4}{\mathbf{1}\ mol\ S} \times material\ L\ (dilution) \times \frac{L}{\mathbf{0{,}07}\ mol\ H_2SO_4} \times \frac{\mathbf{1002}\ g\ H_2SO_4}{L} \times (H_2SO_4\ density) = \boldsymbol{H_2SO_4\ dose}$

3.2.1. Sulphuric acid at 0,07 M

A 0.07 M sulphuric acid solution is prepared to simulate the effluent procedure from the SOB reactor. The reactant sulphuric acid 96% and deionised water are required. Volumetric flasks (1.00, 0.10 and 0.05 L), a pipe, a Pasteur pipe, a beaker, a funnel, and a fume hood are required as an equipment to produce 1 L of 0.07 M sulphuric acid solution. Calculations were made following equations 2, 3, 4 and 5.

Eq. 2 $n = 0{,}07\ M \times 1\ L = 0{,}07\ mol\ H_2SO_4$

Eq. 3 $m = 0{,}07\ mol \times 96 = 6{,}72\ g\ H_2SO_4$

Eq. 4 $\frac{98}{100} = \frac{6{,}72}{X} \rightarrow X = 7\ g\ H_2SO_4$

Eq. 5 $V = \frac{m}{d} = \frac{7\ g\ H_2SO_4}{1{,}84\ g/mL} = 3{,}8\ mL\ H_2SO_4$

3.2.2. Solubilization index

The copper solubilization indexes for each factor’s combination are calculated by the equations 6 and 7.

Eq. 6 $$\frac{(strips)\ Cu\, mg/kg\ dry\ sludge \times (JarTest)\ Diltuion}{(sludge) Density} = Cu\, mg/kg\ clarified$$

Eq. 7 $$Cu\ \ mg/kg\ sludge - Cu\, mg/kg\ clarified = Solubilization\ index$$

3.3. Analytical methods

A brief description of all analytical methods is done in the following paragraphs. The determination of total and volatile solids, conductivity, density, alkalinity and pH follow the methods of Standard Methods (APHA, 2005). The references of other described measurements are included in each section.

3.3.1. pH determination

Calibration solutions (pH 4, pH 7 and pH 9) are required. The equipment required is pH-meter. To obtain the most accurate results, all samples were at room temperature at the time of each test. The pH of the samples and the inside of the reactor was measured using a Crison micro-pH 2000 pH-meter. The device is calibrated every three days.

3.3.2. Conductivity determination

The equipment required is conductivity meter Crison CM 35 which calibration is required (147 µS, 1413 µS and 12,88 mS). All samples were at room temperature at the time of each test. The conductivity meter bult-in door a temperature sensor Pt 1000, used to measure the temperature of each sample.

3.3.3. Solids total and volatile solids determination

No reactant is required. The equipment required are a weighing scale, a crucible per sample, an oven, and a muffle. The procedure starts with a grater and pencil, it is listed the crucibles at the bottom. It is also labelled on the side so when the crucibles are changed from the laboratory oven to the muffle it is easier to recognize the samples. The marker goes out after the muffle. Then, weight the empty crucibles. Without tearing it is added 10 to 20 mL of the sample, previously shaking them. The crucibles are left on the oven (at 105-110 °C), removed 24 hours later, waited to cool down and then weighed again. This will be the weight of the total solids. Next the crucibles are put into the muffle (500 °C) for 3,5 hours, waited to cool down and weighed. The weight of the volatile solids is known. Finally, the crucibles are cleaned (using the same process but without adding samples) and leaving the crucibles into the drain. Total and volatile solids concentration is expressed as mass percentage (%) on wet basis.

3.3.4. Apparent density

The equipment required includes an analytical balance, a volumetric flask and a funnel. No reactant is required. The volumetric flask is tare and the material is added. Then the density of the material is calculated using Eq. 8.

Eq. 8 $$\frac{material\ weight}{material\ volume} = density$$

3.3.5. Alkalinity determination

A solution of sulphuric acid 0.05 M is used as a reactant. The equipment required are burette, a beaker, an agitator and a pH-meter. First the sample is centrifugated at 2,200 rpm for 10 minutes; then, 10 mL of the supernatant is used. Then pH is measured and is being added a known volume of the sulphuric acid. The sample must be constantly stirring. Every time that the acid is added the pH is measured. The interesting values to know the alkalinity are the volume of sulphuric acid addition closer to pH 5,75 and 4,3.

3.3.6. Acid-base titration

The acid-base titration was used to analyse the H_2SO_4 concentration of the reactor effluent. A 0.2 M NaOH is used as reagent and a beaker, a burette, a pipette, a stirrer, and a pH-meter. The procedure is: (i) the 0.2 M NaOH solution is made and introduced into the burette; (ii) 25 mL of the effluent is added at the beaker and the pH is measured by stirring the sample; (iii) known volumes of the NaOH solution are added into the beaker with the effluent and pH is measured. At the time that the solution into the beaker reaches or exceeds pH 7, it means that all H_2SO_4 will have been neutralized. If it is necessary, the exact volume of NaOH used to reach pH 7 is calculated. Then the H_2SO_4 concentration in the effluent is calculated using Eq. 9.

Eq. 9 $$\frac{V_{(NaOH)} \times M_{(NaOH)}}{V_{(H_2SO_4)}} = M_{(H_2SO_4)}$$

3.3.7. Copper determination

The copper was chosen as the reference of HM. The strips MQuant Copper Test are used to measure the Cu concentration in the sludge and clarified supernatants after the settling period at the end of the Jar-Test. Samples should be between 15 °C and 25 °C and with a pH between 2 and 6 when the strips are used. Once temperature and pH has been adjusted, the procedure for measuring Cu is: (i) insert the reaction zone of the strip during 1 second; (ii) the excess is removed from strip by shaking it; (iii) wait 30 seconds; (iv) the colour of the strip is classified according to the colour of the label (Figure 6); (v) the corresponding value of Cu in mg/L is read.

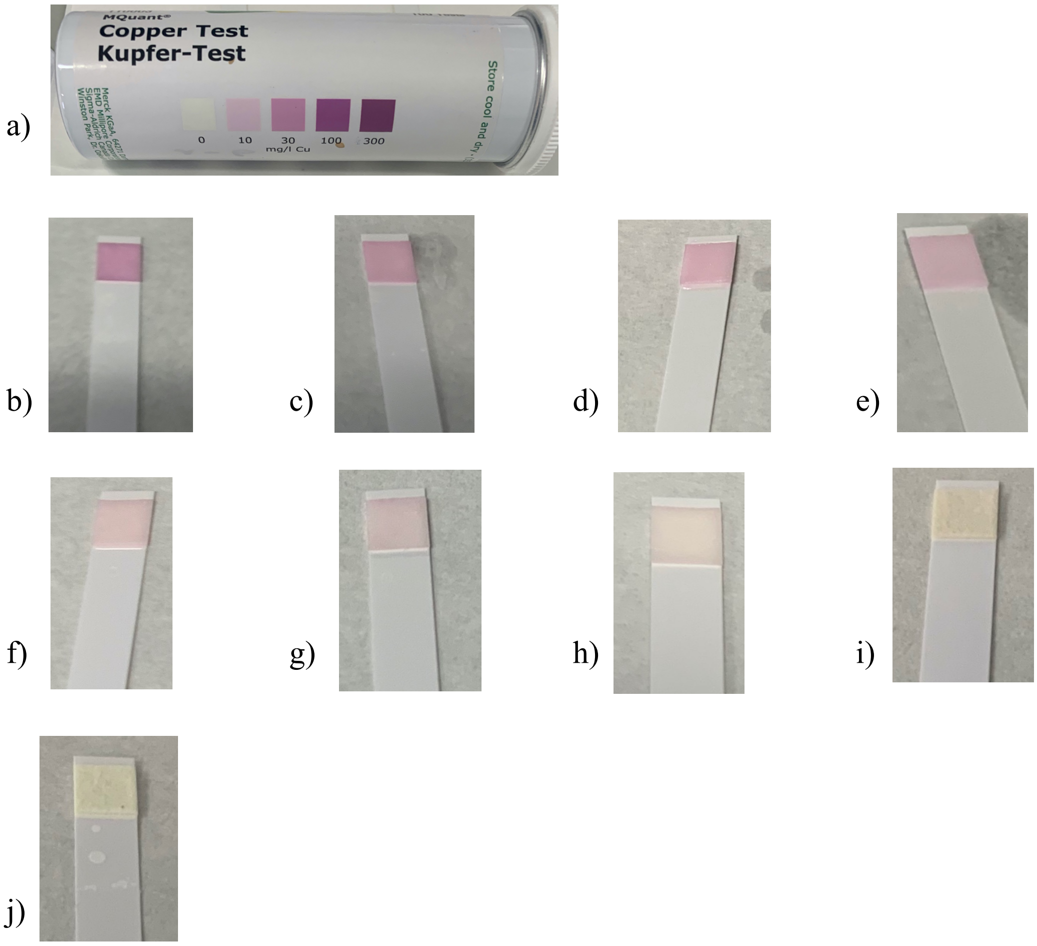

Figure 6. Copper strip classification by colour. ***a)*** *Label with the colours classification;* ***b)*** *strip that analyses 30 mg/L;* ***c)*** *strip that analyses 25 mg/L;* ***d)*** *strip that analyses 20 mg/L;* ***e)*** *strip that analyses 15 mg/L;* ***f)*** *strip that analyses 10 mg/L;* ***g)*** *strip that analyses 7,5 mg/L;* ***h)*** *strip that analyses 5 mg/L;* ***i)*** *strip that analyses 2,5 mg/L;* ***j)*** *strip that analyses 0 mg/L.*

To rise the pH of clarified samples, reagent sodium acetate (see Table 5) is used. A weighing scale, a spatula, a beaker, a stirred, and a pH-meter are used as equipment. The necessary sodium acetate weights for each clarified are calculated experimentally. First, the pH of the sample is measured. Then the adequate sodium acetate is added according to the Table 5. Then the solution is left stirring until all the sodium acetate has reacted and the pH is measured again. If a pH above 2 has not been reached yet, more sodium acetate is added. To decrease the pH of samples till pH 6, a digestion with royal water (UNE-EN 13650, 2002) was used.

Table 5. Sodium acetate necessary to raise the pH of clarified samples

Jar	Dilution	H_2SO_4 (g)	Initial pH	$C_2H_3NaO_2$ (g)	Final pH
1	1	152	0.27 – 0.56	1.5 – 1.9	2.05 – 3.30
2	1/2	76	0.43 – 0.74	1.0 – 1.3	2.14 – 3.30
3	1/10	15	0.95 – 1.34	0.25 – 0.30	2.56 – 3.60
4	1	455	0.14 – 0.33	3.0 – 3.50	2.02 – 3.24
5	1/2	228	0.22 – 0.69	2.25 – 2.75	2.66 – 3.35
6	1/10	46	0.46 – 0.72	0.60 – 0.75	2.50 – 3.94

For the total Cu concentration in the sludge, a hydrolysis was done. The reagents required are concentrated hydrochloric acid, concentrated nitric acid, deionised water, and sodium acetate. A weighting scale, a pipette, a 100 mL flask, a funnel, a heater, a thermometer, a filtering equipment, a crucible, and an oven are required as equipment. Also, part of the process must be done under a fume hood. First, samples are dried at 105ºC (similarly to section 1.6.3.) and 1 g of dry and milled sample is weighted and inserted into a 100 mL flask. Then, 1 mL of deionised water, 21 mL of concentrated hydrochloric acid and 7 mL concentrated nitric acid are added. The solution is left for 16 hours. Glass balls are added into the flask (to avoid the foams generation). The flask is put on a heater covered with a glass funnel. It is kept boiling for 2 hours. The digested is made up to the mark and then is vacuum filtered with a filter paper without phosphorus. Cu can be analysed in the digestate. As the pH of the digestate is below 2, it is necessary to raise it as described previously.

4. Results and Discussions

4.1. SOB reactor operation

4.1.1. SOBs activation and reactor inoculation

The SOBs that had been used in the previous work (Tobias, 2018) were intended to inoculate the reactor, but 2 years had passed, without renewing the medium, that is why it wasn't known if the SOBs would remain actives. Thereby, the 7 chosen flasks were introduced in a falcon tube with new medium (S1). Also, a follow-up of the pH decrease was made (see Table 6). The change of medium was made on February 17^{th}.

Table 6 pH value of 2018-cultures. Notes: the identification of flasks was this: C-: negative control made at 02/03/18; C+: positive control made at 02/03/18; M0: negative control made at 17/02/20; M1: 50 mL of SOB medium + 1 mL of second subculture of mixed inoculum + 0,5 g of S°; M2: 4/1/1; M3: 3/1/2; M4: 4/2/1; M5: 4/2/2; M6: 16/1/1; M7: 16/2/1.

Flask ID.	2018-Cultures pH	Falcon tube (17/02/20) pH	Falcon tube (28/02/20) pH	Falcon tube (05/03/20) pH	Falcon tube (01/07/20) pH
C-	-	4-5	4-5	4-5	4-5
C+	0	1	1	1	1
M0	4-5	4-5	4-5	4-5	4-5
M1	**1**	**3-4**	**2-3**	**2-3**	**1**
M2	**0-1**	**2-3**	**2-3**	**2-3**	**1**
M3	0-1	2-3	2-3	2-3	2-3
M4	**0-1**	**2-3**	**2**	**2**	**1**
M5	**0-1**	**1-2**	**1-2**	**1-2**	**1**
M6	3-4	4-5	4-5	4-5	4-5
M7	3-4	4-5	4-5	4-5	4-5

On July 1^{st}, a significant decrease of the pH was observed in 4 of the 7 flasks. These flasks are: M1, M2, M4 and M5, meaning that the microorganisms are alive and working. So, these 4 cultures were being inoculated into the reactor with the pure culture of *Acidithiobacillus thiooxidans*.

Also, a follow-up of the second source to inoculate into the reactor was made (Table 7). S2 was begun to activate on July 10^{th} and on August 4^{th} was inoculated into the reactor, since the pH decreased.

Table 7. pH value of the second source activation. Notes: the identification of flasks was this: C-: negative control made at 02/03/18; C+: positive control made at 02/03/18; M1: 50 mL of SOB medium + 1 mL of second subculture of mixed inoculum + 0,5 g of S°; M2: 4/1/1; M4: 4/2/1; M5: 4/2/2.

Flask ID.	Bottle (10/07) pH	Bottle (20/07) pH	Bottle (27/07) pH	Bottle (03/08) pH
C-	4-5	4-5	4-5	4-5
C+	3-4	3-4	3-4	3
M1	4	3	2-3	2
M2	4	3-4	3	2-3
M4	3-4	3	3	2
M5	3-4	3	3	2
Pure culture	4	3	2-3	2

4.1.2. Reactor monitoring

The continuous operation started on August 10th and the feeding bottle was refilled every Monday, Wednesday and Friday.

The Jar-Test was operating with a sulphuric acid concentration at 0.07 M and the average sulphuric acid concentration of the reactor effluent is 0.06 M and an average pH of 2.13. It means that, the results of the Jar-Test may be similar to those that can be obtained by using the reactor effluent.

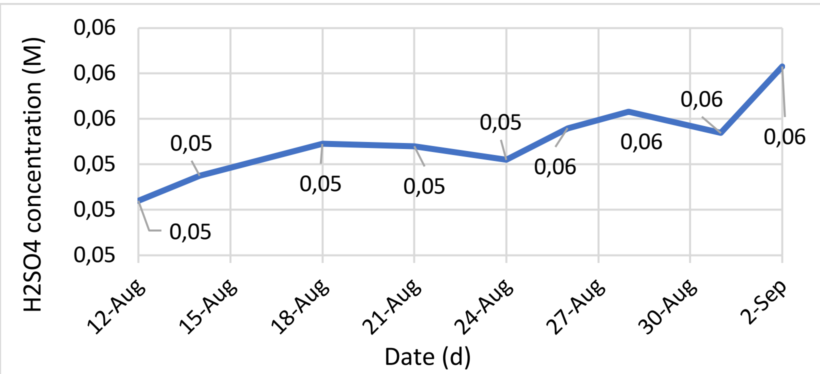

Figure 7. Sulphuric acid concentration of the reactor effluent during the continuous operation.

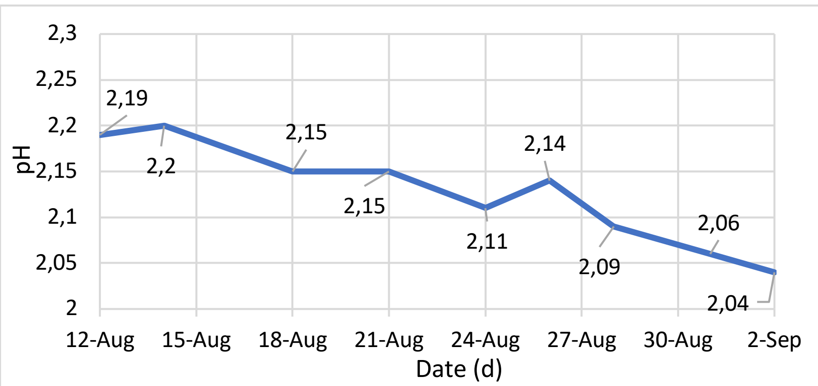

Figure 8. pH of the reactor effluent during the continuous operation.

According to the Figure 7, there was an increase of the sulphuric acid concentration in the effluent. At the same time, according to the increase of the acid concentration there was a decrease of pH (Figure 8).

4.2. Bioleaching test

4.2.1. Sludge characterization

The sludge characterization was made at the beginning of each Jar-Test and 2 replicas every time. Table 8 shows the average of the sludge analyses.

Table 8. Sludge characterization

Total Solids (%)	Conductivity (Sm)	pH	Density (g/l)	Alkalinity (g $CaCO_3$/l)	Cu (mg/kg)
2.0051 ± 0.1306	9.93 ± 0.61	8.27 ± 0.17	1.004 ± 0.0014	4.483 ± 0.103	207.75 ± 5.65

4.2.2. Jar-Test doses

Jar-Test was started on July 20th until August 24th. In Table 9 there is a summary of factor combination (Material dilution, Sulphuric acid dose and Stirring time) in the Jar-Test. There were 3 replicas for each factor combination.

Table 9 Factor combination summary.

Factor Combination ID.	Material Dilution	Sulphuric Acid Dose (expressed in g S°/l)	Stirring Time (days)
FC 1	1	1	3
FC 2	1/2	1	3
FC 3	1/10	1	3
FC 4	1	3	3
FC 5	1/2	3	3
FC 6	1/10	3	3
FC 7	1	1	6
FC 8	1/2	1	6
FC 9	1/10	1	6
FC 10	1	3	6
FC 11	1/2	3	6
FC 12	1/10	3	6

Different solutions of sewage sludge were made to modify the total solids content. As the total volume of the jar is 1 L and considering that the dose of sulphuric acid should be added, the useful volume of the material was set at 0.35 L. The material and water weight were calculated (Table 10) for each dilution and the sulphuric acid solution doses are in Table 11.

Table 10 Material and water weight.

Dilution	Material (g)	Water (g)
1	351.05	0
1/2	175.53	175
1/10	35.11	315

Table 11 H_2SO_4 dose (g H_2SO_4 solution at 0.07 M).

	Sulphuric Acid Solution Dose (g)	
Dilution	1 g S^0/L	3 g S^0/L
1	152	455
1/2	76	228
1/10	15	46

4.2.3. Jar-Test monitoring

Conductivity, pH and weight were measured daily (Monday to Friday) for each jar.

There was no significant difference in the weightless (Figure 9), by water evaporation. At higher dilution could result in higher weightless, but bearing in mind that total solids of the sludge are 2 % (Table 8) is to be expected.

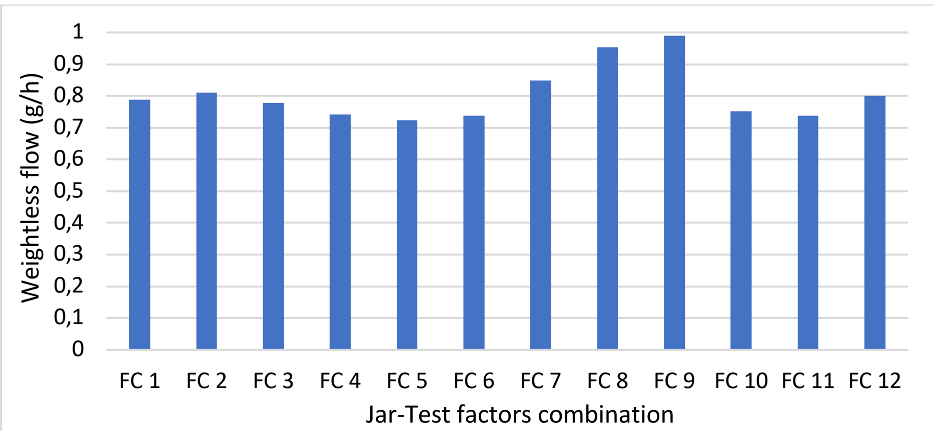

*Figure 9. pH evolution in each factor combination during Jar-Test. **FC 1** (factor combination 1): Dilution 1, 1 g S°/L, 3 days; **FC 2**: Dilution ½, 1 g S°/L, 3 days; **FC 3**: Dilution 1/10, 1 g S°/L, 3 days; **FC 4**: Dilution 1, 3 g S°/L, 3 days; **FC 5**: Dilution ½, 3 g S°/L, 3 days; **FC 6**: Dilution 1/10, 3 g S°/L, 3 days; **FC 7**: Dilution 1, 1 g S°/L, 6 days; **FC 8**: Dilution ½, 1 g S°/L, 6 days; **FC 9**: Dilution 1/10, 1 g S°/L, 6 days; **FC 10**: Dilution 1, 3 g S°/L, 6 days; **FC 11**: Dilution ½, 3 g S°/L, 6 days; **FC 12**: Dilution 1/10, 3 g S°/L, 6 days.*

The evolution of pH for each Jar-Test and factors combination can be seen in Figure 10. These results indicate that during Jar-Test there is an increase in pH higher in Jars with 6 days of stirring than 3 days. It could mean, that a part the sulphuric acid is reacting, probably with heavy metals and some other components of the mixture.

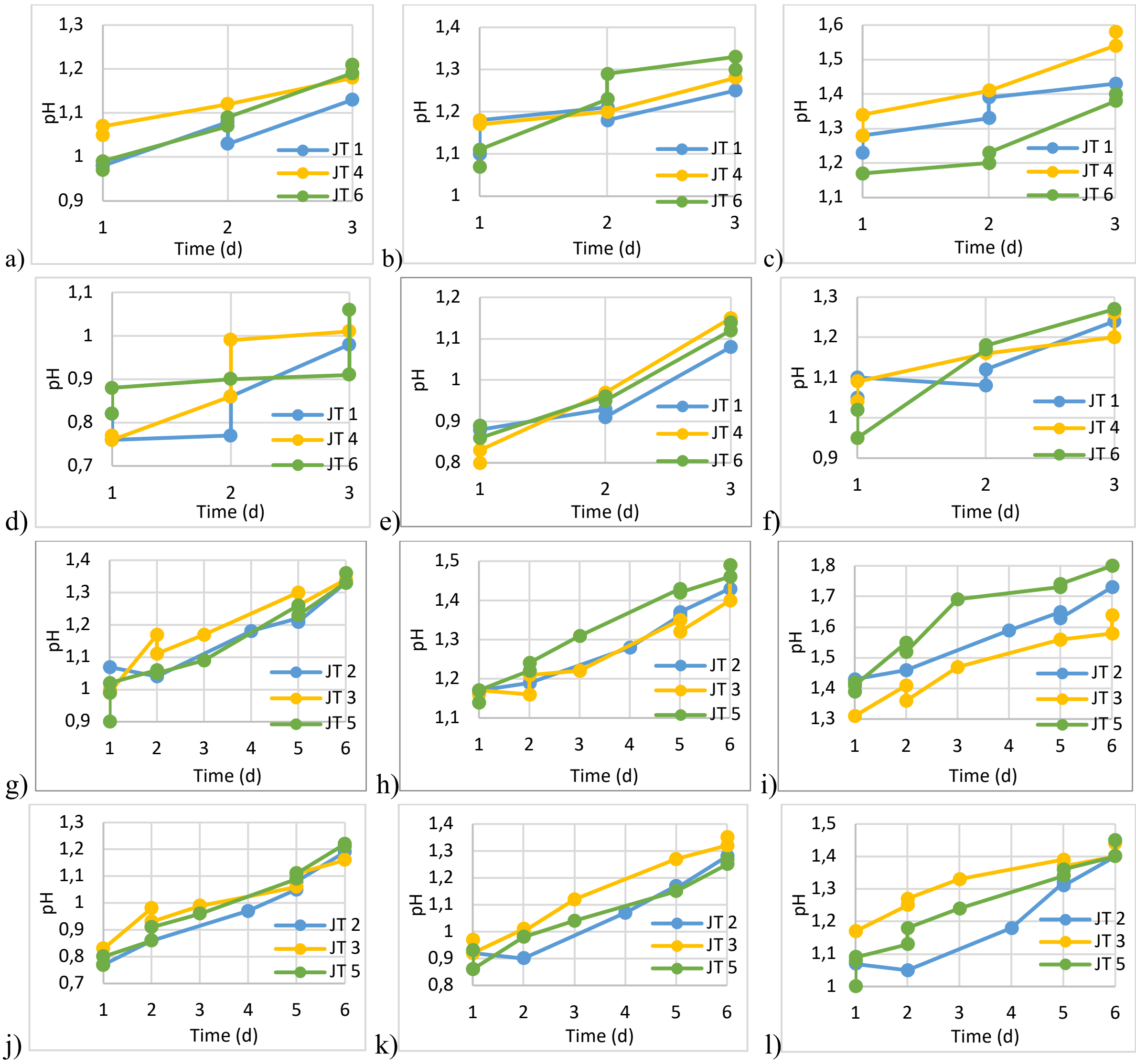

*Figure 10. pH evolution in each factor combination during Jar-Test. **a)** Factor combination (FC 1): Dilution 1, 1 g S°/L, 3 days; **b)** FC 2: Dilution ½, 1 g S°/L, 3 days; **c)** FC 3: Dilution 1/10, 1 g S°/L, 3 days; **d)** FC 4: Dilution 1, 3 g S°/L, 3 days; **e)** FC 5: Dilution ½, 3 g S°/L, 3 days; **f)** FC 6: Dilution 1/10, 3 g S°/L, 3 days; **g)** FC 7: Dilution 1, 1 g S°/L, 6 days; **h)** FC 8: Dilution ½, 1 g S°/L, 6 days; **i)** FC 9: Dilution 1/10, 1 g S°/L, 6 days; **j)** FC 10: Dilution 1, 3 g S°/L, 6 days; **k)** FC 11: Dilution ½, 3 g S°/L, 6 days; **l)** FC 12: Dilution 1/10, 3 g S°/L, 6 days. **Legend**: JT 1: Jart-Test 1; JT 2: Jart-Test 2; JT 3: Jart-Test 3; JT 4: Jart-Test 4; JT 5: Jart-Test 5; JT 6: Jart-Test 6.*

4.2.4. Copper solubilization index

A synthesis of the clarified results, Cu solubilization, conductivity and pH can be seen in Table 12. Also, total solids content was measured, but because of the acid in the samples the results are not analysable.

*Table 12. Cu solubilization index, conductivity and pH in the clarified for each factor combination. **FC 1**: Dilution 1, 1 g S°/L, 3 days; **FC 2**: Dilution ½, 1 g S°/L, 3 days; **FC 3**: Dilution 1/10, 1 g S°/L, 3 days; **FC 4**: Dilution 1, 3 g S°/L, 3 days; **FC 5**: Dilution ½, 3 g S°/L, 3 days; **FC 6**: Dilution 1/10, 3 g S°/L, 3 days; **FC 7**: Dilution 1, 1 g S°/L, 6 days; **FC 8**: Dilution ½, 1 g S°/L, 6 days; **FC 9**: Dilution 1/10, 1 g S°/L, 6 days; **FC 10**: Dilution 1, 3 g S°/L, 6 days; **FC 11**: Dilution ½, 3 g S°/L, 6 days; **FC 12**: Dilution 1/10, 3 g S°/L, 6 days.*

Factors Combination (fc)	Cu Solubilization (mg Cu/kg sludge)	Conductivity (Sm)	pH
FC 6	148.25	1506.33	1.38
FC 5	159.87	1540.33	1.18
FC 2	169.83	628.00	1.43
FC 3	173.15	672.33	1.74
FC 4	186.43	1154.67	1.05
FC 1	188.09	541.07	1.23
FC 9	189.75	755.67	1.83
FC 12	189.75	2062.67	1.60
FC 8	191.41	716.53	1.54
FC 11	194.73	1979.33	1.38
FC 7	195.56	688.60	1.32
FC 10	203.86	1299.33	1.18

No relation between conductivity and Cu solubilization has been found (Figure 11), probably, because sulphuric acid interferes, since this compound has a high a conductivity. Also, this explanation can be seen in Table 12, clarified with a higher conductivity are those which the sulphuric acid addition is 3 g S°/L.

Besides pH in the clarified has no relation either with the Cu solubilization index (Figure 12), since pH is affected too by sulphuric acid concentration. However, if the pH of clarified is compared to each factor, we can see that samples which had a stirring time of 6 days get a more basic pH than their homologous sample with a stirring time of 3 days. Could mean that in 6 days more sulphuric acid had been reacted with heavy metals, solubilizing them. Also, those clarified that sulphuric acid factor is 3 g S°/L obtained a lower pH than their homologous with a sulphuric acid factor of 1 g S°/L.

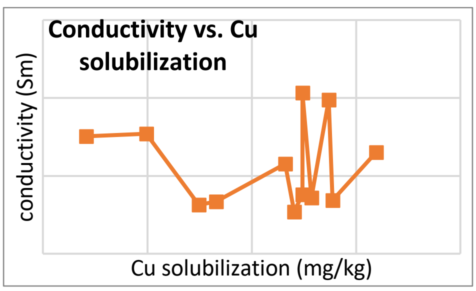

Figure 11. Relation between conductivity and Cu solubilization in the clarified.

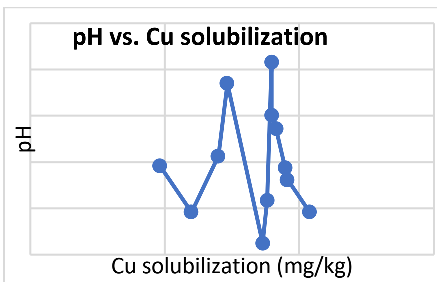

Figure 12. Relation between pH and Cu solubilization in the clarified.

In Figure 13 there is the comparison between the stirring time and Cu solubilization index. It is clearly seen as the jars that have had a stirring time of 6 days have a higher Cu solubilization index (189.75 mg Cu/kg sludge – 203.86 mg Cu/kg sludge) than jars that have had 3 days of stirring time (148.25 mg Cu/kg sludge – 188.09 mg Cu/kg sludge).

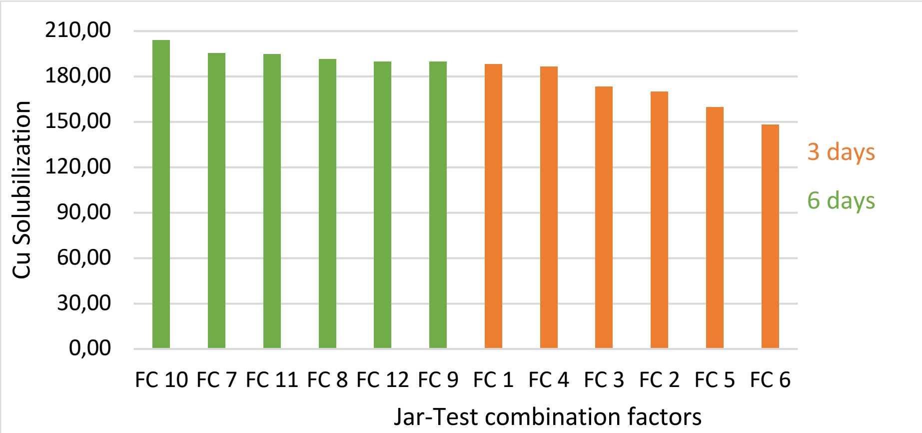

*Figure 13. Cu solubilization as a function of stirring time factor. **FC 1** (factor combination 1): Dilution 1, 1 g S°/L, 3 days; **FC 2**: Dilution ½, 1 g S°/L, 3 days; **FC 3**: Dilution 1/10, 1 g S°/L, 3 days; **FC 4**: Dilution 1, 3 g S°/L, 3 days; **FC 5**: Dilution ½, 3 g S°/L, 3 days; **FC 6**: Dilution 1/10, 3 g S°/L, 3 days; **FC 7**: Dilution 1, 1 g S°/L, 6 days; **FC 8**: Dilution ½, 1 g S°/L, 6 days; **FC 9**: Dilution 1/10, 1 g S°/L, 6 days; **FC 10**: Dilution 1, 3 g S°/L, 6 days; **FC 11**: Dilution ½, 3 g S°/L, 6 days; **FC 12**: Dilution 1/10, 3 g S°/L, 6 days.*

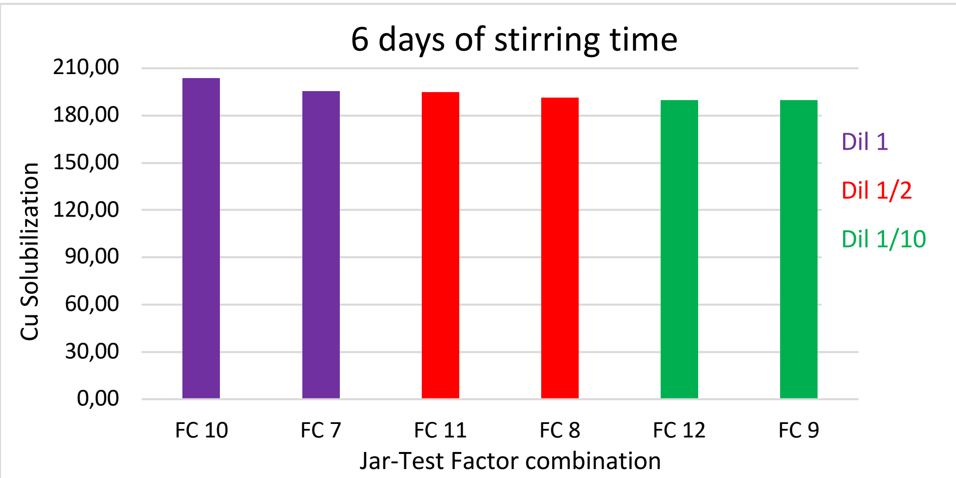

*Figure 14. Cu solubilization as a function of material dilution factor with 6 days of stirring time. **FC 7** (factor combination 7): Dilution 1, 1 g S°/L, 6 days; **FC 8**: Dilution ½, 1 g S°/L, 6 days; **FC 9**: Dilution 1/10, 1 g S°/L, 6 days; **FC 10**: Dilution 1, 3 g S°/L, 6 days; **FC 11**: Dilution ½, 3 g S°/L, 6 days; **FC 12**: Dilution 1/10, 3 g S°/L, 6 days.*

Both, in Figure 14 and Figure 15 show that the variant that has obtained better results of Cu solubilization in the material dilution factor is that one that had no dilution (Dil. 1) (186.43 mg Cu/kg sludge – 203.86 mg Cu/kg sludge).

The second variant of the material dilution factor that have had a better Cu solubilization is dilution ½ (159.87 mg Cu/kg sludge – 193.73 mg Cu/kg sludge), followed by dilution 1/10 (148.25 mg Cu/kg sludge – 189.75 mg Cu/kg sludge). Although, it seems that with 3 days of stirring time FC 3 (Dilution 1/10, 1 g S°/L, 3 days) have better results than material dilution ½ (Figure 15), it is not met by looking at the overall results.

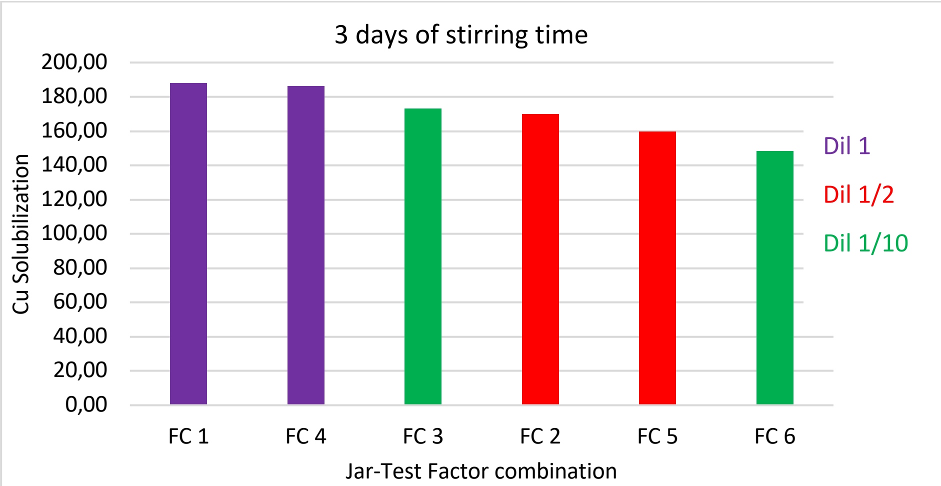

*Figure 15. Cu solubilization as a function of material dilution factor with 3 days of stirring time. **FC 1** (factor combination 1): Dilution 1, 1 g S°/L, 3 days; **FC 2**: Dilution ½, 1 g S°/L, 3 days; **FC 3**: Dilution 1/10, 1 g S°/L, 3 days; **FC 4**: Dilution 1, 3 g S°/L, 3 days; **FC 5**: Dilution ½, 3 g S°/L, 3 days; **FC 6**: Dilution 1/10, 3 g S°/L, 3 days.*

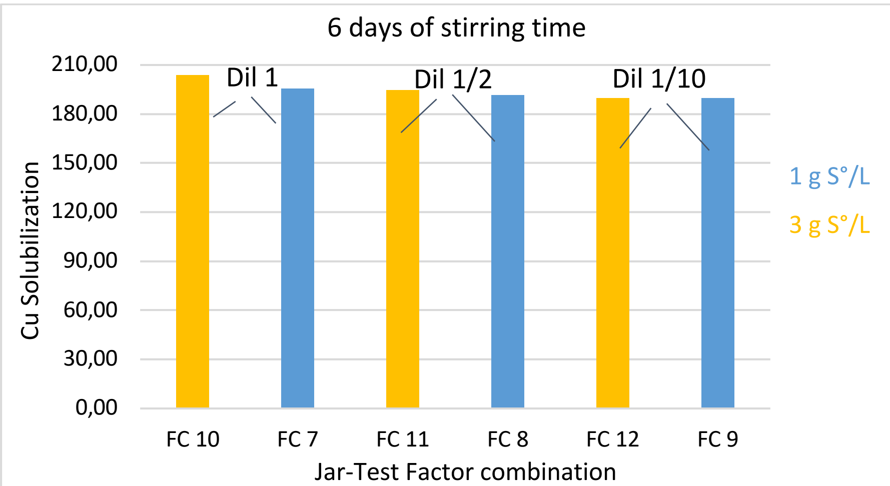

*Figure 16. Cu solubilization as a function of acid sulphuric dose factor. **FC 7** (factor combination 7): Dilution 1, 1 g S°/L, 6 days; **FC 8**: Dilution ½, 1 g S°/L, 6 days; **FC 9**: Dilution 1/10, 1 g S°/L, 6 days; **FC 10**: Dilution 1, 3 g S°/L, 6 days; **FC 11**: Dilution ½, 3 g S°/L, 6 days; **FC 12**: Dilution 1/10, 3 g S°/L, 6 days.*

According to Figure 16 for the jars with 6 days of stirring time and for each material dilution, Cu solubilization it is higher in those with a 3 g S°/L of sulphuric acid dose (189.75 mg Cu/kg sludge – 203.86 mg Cu/kg sludge) than those with a 1 g S°/L of sulphuric acid dose (189.75 mg Cu/kg sludge – 195.56 mg Cu/kg sludge).

On the other hand, according to Figure 17, it is the opposite for the jars with 3 days of stirring time and for each material dilution. In this case, those which 1 g S°/L of sulphuric acid dose have a higher Cu solubilization (169.83 mg Cu/kg sludge – 188.09 mg Cu/kg sludge) than those with 3 g S°/L of sulphuric acid dose (148.25 mg Cu/kg sludge – 186.43 mg Cu/kg sludge).

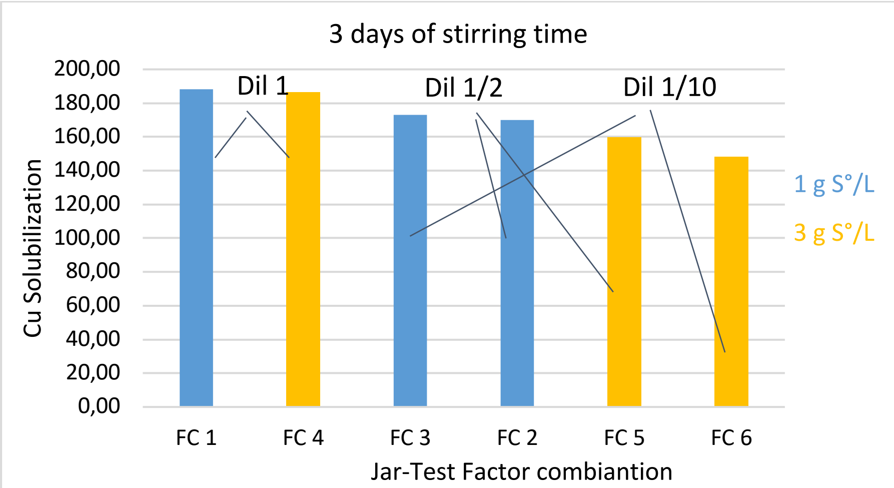

Figure 17. Cu solubilization as a function of acid sulphuric dose factor. ***FC 1*** *(factor combination 1): Dilution 1, 1 g S°/L, 3 days;* ***FC 2****: Dilution ½, 1 g S°/L, 3 days;* ***FC 3****: Dilution 1/10, 1 g S°/L, 3 days;* ***FC 4****: Dilution 1, 3 g S°/L, 3 days;* ***FC 5****: Dilution ½, 3 g S°/L, 3 days;* ***FC 6****: Dilution 1/10, 3 g S°/L, 3 days.*

5. Conclusions

According to the results, it can be concluded that a copper solubilization can be achieved with a sulphuric acid solution which simulates the effluent from a reactor inoculated with sulphur-oxidizing microorganisms.

Also, it has been achieved to set in motion a continuous operation of a reactor inoculated with sulphur-oxidizing microorganisms.

First of all, it was confirmed that the sulphur-oxidizing microorganisms (S2) to be used to inoculate the reactor could be activated, since by feeding them with new medium (February 17th), the initial pH (2-4) was decreased (pH 1 on July 1st), confirming that microorganisms were transforming the sulphur into sulphuric acid.

A continuous stirred tank reactor (CSTR) was mounted and launched, with some adjustments during the continuous operation. The results obtained from the effluent, a pH decrease from 2.19 to 2.04 and sulphuric acid concentration increase from 0.05 M to 0.06 M, were confirmed that the microorganisms were working properly. Even though, 0.06 M it cannot be taken as the final sulphuric acid concentration in the effluent, since the reactor was working in the continuous operation 24 days and sulphur-oxidizing bacteria need more than a month to grow.

As for the Jar-Test, according to the results there was solubilization of the copper (148.25 mg Cu/kg sludge – 203.86 mg Cu/kg). During Jar-Test, pH of the Jars, were increasing through the operational time, demonstrating that sulphuric acid was transforming into sulphate.

Find the best factors combination was what was intended doing a Jar-Test. The factor combination that obtained the best Cu solubilization is: (FC 10) Dilution 1 of the material, 6 days of stirring time and 3 g S°/L of the sulphuric acid dose (203.86 mg Cu/kg sludge).

- Material dilution. No dilution is needed to get a properly Cu solubilization, probably, because the sludge used has a low total solids content, (2%). If another material with a higher total solids content is used, could be necessary a dilution of the material to get, for example, 2% of total solids content.

- Stirring time. All the results show that 6 days of stirring time achieve a higher Cu solubilization than 3 days of stirring time. It means that 3 days of stirring time it is not enough time to obtain a properly Cu solubilization.

- Sulphuric acid dose. It seems that when the stirring time is 6 days, 3 g S°/L of sulphuric acid dose gets better Cu solubilization. But, it is 1 g S°/L of sulphuric acid dose which achieves higher Cu solubilization when the stirring time is 3 days. Even though, as 6 days of stirring time is better than 3 days, the conclusion is that 3 g S°/L of sulphuric acid dose it has to be chosen.

5.1. Future works

- Next step should be to analyse, in addition to copper, all others heavy metals that can be a problem such as copper.
- Also, a Jar-Test using other types of materials subject to become compost could be realized, such as digested FORM.
- Finally, a test with the effluent of the reactor with sulphur-oxidizing microorganisms must be tested.

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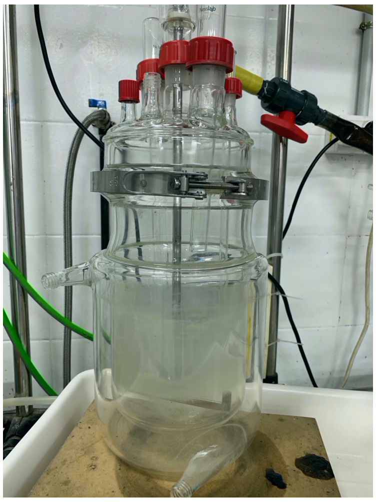
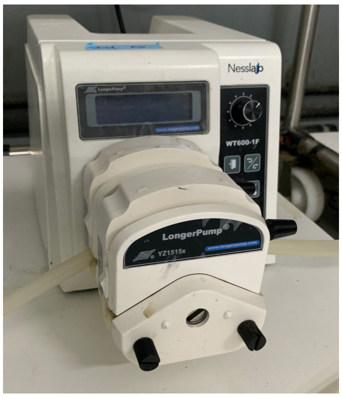
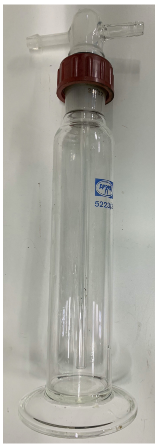
Annexes

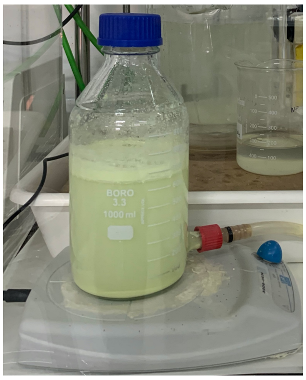
Annex 1. Parts of the reactor. a) Pyrex reactor; b) Peristaltic pump; c) Trap water; d) Air pump; e) Feed bottle in an agitator; f) Effluent storage.

a) 

b) 

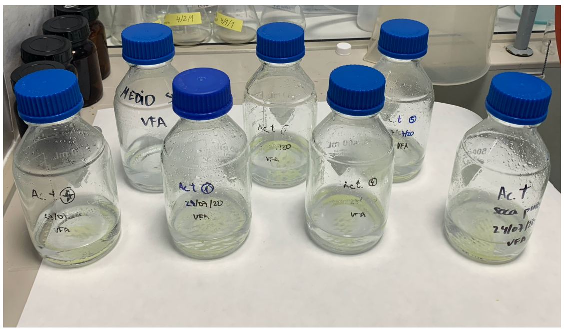
c) 

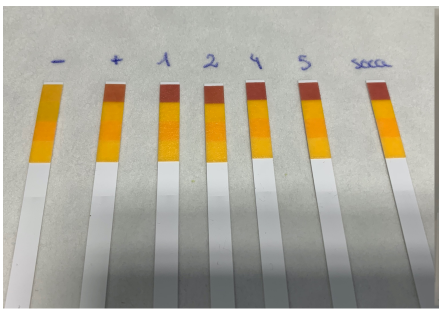
d) 

e) 

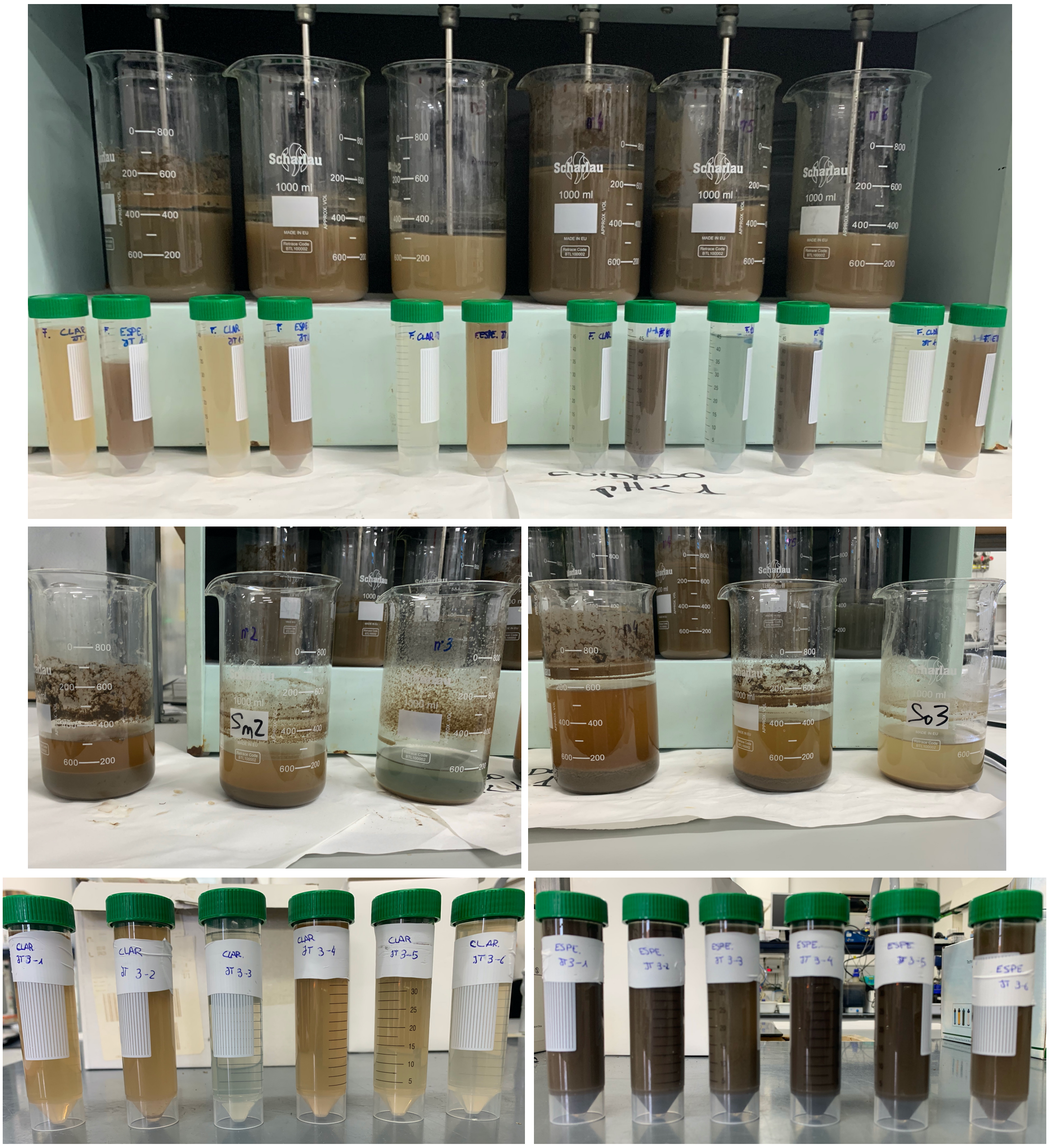
f) 

Annex 2. Source 2 activation and pH strips.





Annex 3. Clarified and settled.



Annex. 4 Sludge characterization.

	Total solids (%)	Conductivity (Sm)	pH	Density (g/L)	Alcalinidad (g $CaCO_3$/L)	Cu (mg Cu/kg sludge)
			Jar-Test 1			
Replica 1	2.1074	10.29	8.13	1.003	4.5	213,67
Replica 2	1.9151	10.9	8.46	1.002	4.5	
Total	2.0113	10.595	8.295	1.002	4.5	
			Jar-Test 2			
Replica 1	1,8007	10,46	8,2	1,004	4,6	211,97
Replica 2	1,9087	10,97	8,41	1,006	4,6	
Total	1,8547	10,715	8,305	1,005	4,6	
			Jar-Test 3			
Replica 1	1,8596	9,52	7,96	1,002	4	207,95
Replica 2	2,0263	9,82	8,1	1,002	4,6	
Total	1,9429	9,67	8,03	1,002	4,3	
			Jar-Test 4			
Replica 1	2,0593	9,68	8,29	1,005	4,3	202,02
Replica 2	2,0328	10,13	7,92	1,004	4,7	
Total	2,0461	9,905	8,105	1,004	4,5	
			Jar-Test 5			
Replica 1	2,0136	9,28	8,51	1,006	4,5	200,57
Replica 2	1,8670	9,19	8,44	1,004	4,4	
Total	1,9403	9,235	8,475	1,005	4,45	
			Jar-Test 6			
Replica 1	2,2283	9,43	8,46	1,005	4,5	201,93
Replica 2	2,2418	9,49	8,32	1,005	4,6	
Total	2,2351	9,46	8,39	1,005	4,55	

Annex 5. Jar-Test monitoring

Material dilution 1		**Sulphuric acid dose 1 g S°/L**		**Stirring time 3**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 1		
1	784,95	0,98	25,4	221	
1	770,37	0,98	24,9	302	
2	752,33	1,08	25,3	316	
2	749,55	1,03	25,6	317	
3	734,04	1,13	24,7	86,2	1/6
			Jar-Test 4		
1	778,18	1,05	26	61,5	1/10
1	776,76	1,07	25,8	112,5	1/6
2	774,71	1,12	25,3	109,1	1/6
3	743,61	1,18	25,2	92,9	1/6
3	739,46	1,21	24,8	100,3	1/6
			Jar-Test 6		
1	772,99	0,97	25,3	56,2	1/10
1	776,61	0,99	23,8	62,1	1/10
2	771,59	1,07	24,7	56,4	1/10
2	754,6	1,09	24,4	57,8	1/10
3	750,03	1,19	25,4	60	1/10
3	735,05	1,21	24,5	61,4	1/10

Material dilution 1/2		**Sulphuric acid dose 1 g S°/L**		**Stirring time 3**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 1		
1	716,15	1,1	25	208	
1	699,76	1,18	24,4	233	
2	682,59	1,21	25,1	239	
2	679,87	1,18	25,1	235	
3	663,15	1,25	24,3	66,3	1/6
			Jar-Test 4		
1	708,15	1,18	25,7	55,2	1/10
1	701,33	1,17	25,4	101,5	1/6
2	699,83	1,2	25,1	99,3	1/6
3	665,57	1,28	24,9	64,6	1/6
3	660,84	1,33	24,4	57,3	1/6
			Jar-Test 6		
1	694,75	1,07	25,4	34	1/10
1	702,37	1,11	23,5	35	1/10
2	695,85	1,23	24,6	35,9	1/10
2	679,87	1,29	24,2	36,2	1/10
3	674,89	1,33	25,1	35,5	1/10
3	658,1	1,3	24,2	37,7	1/10

Material dilution 1/10		**Sulphuric acid dose 1 g S°/L**		**Stirring time 3**	
Day	Weight (g)	pH	Temperature (°C)	Conductivity (sm)	Dilution
			Jar-Test 1		
1	653,97	1,23	24,6	65,3	
1	639,38	1,28	24	95,2	
2	622,45	1,33	24,7	93,8	
2	619,83	1,39	24,6	95,4	
3	603,1	1,43	23,9	26,3	1/6
			Jar-Test 4		
1	647,82	1,28	25,5	18,22	1/10
1	646,37	1,34	25,2	35	1/6
2	644,4	1,41	24,9	34,9	1/6
3	608,7	1,54	24,8	77,7	
3	605,43	1,58	24,2	21,9	1/6
			Jar-Test 6		
1	638,04	1,17	25	71,6	
1	646,87	1,17	23,7	72	
2	642,79	1,2	24,6	68,6	
2	624,75	1,23	24	64,8	
3	620,81	1,38	25,4	62,6	
3	604,76	1,4	24,1	61,2	
Material dilution 1		**Sulphuric acid dose 3 g S°/L**		**Stirring time 3**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 1		
1	1097,71	0,82	25,8	373	
1	1079	0,76	25,6	166,1	1/6
2	1062,2	0,77	25,9	168,3	1/6
2	1058,59	0,86	26,2	164,5	1/6
3	1044,82	0,98	25,3	157,9	1/6
			Jar-Test 4		
1	1087,65	0,77	26,5	91,3	1/10
1	1076,86	0,76	26,3	122,6	1/10
2	1074,03	0,86	25,6	120,1	1/10
3	1044,09	0,99	25,9	153,3	1/10
3	1040,77	1,01	25,2	161	1/6
			Jar-Test 6		
1	1073,98	0,82	27	113,4	1/10
1	1076,88	0,88	24,3	123,9	1/10
2	1070,6	0,9	25,1	116,8	1/10
2	1056,61	0,91	24,9	122,9	1/10
3	1052,29	1,06	25,9	122,5	1/10
3	1038,12	1,04	25,2	127,9	1/10
Material dilution 1/2		**Sulphuric acid dose 3 g S°/L**		**Stirring time 3**	

Day	Weight (g)	pH	Temperature (°C)	Conductivity (sm)	Dilution
			Jar-Test 1		
1	865,65	0,89	25,6	358	
1	853,21	0,88	25,2	125,2	1/6
2	836,88	0,93	25,5	122,3	1/6
2	833,46	0,91	25,9	133,2	1/6
3	819,29	1,08	25	135	1/6
			Jar-Test 4		
1	862,27	0,8	26,2	66,8	1/10
1	850,72	0,83	25,9	54,6	1/10
2	847,29	0,97	25,4	52,8	1/10
3	817,37	1,15	25,6	84,8	1/10
3	814,32	1,14	24,9	140,5	1/6
			Jar-Test 6		
1	847,12	0,8	26,2	66,8	1/10
1	850,72	0,83	25,9	54,6	1/10
2	847,29	0,97	25,4	52,8	1/10
2	817,37	1,15	25,6	84,8	1/10
3	814,32	1,14	24,9	140,5	1/6
3	808,19	1,14	24,8	84,5	1/10

Material dilution 1/10		**Sulphuric acid dose 3 g S°/L**		**Stirring time 3**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 1		
1	673,48	1,05	25,4	190,7	
1	655,4	1,1	24,6	222	
2	641,5	1,08	25,2	229	
2	639,3	1,12	25,4	229,5	
3	626,14	1,24	24,5	50,4	1/6
			Jar-Test 4		
1	667,82	1,04	25,8	22,4	1/10
1	660,68	1,09	25,7	69,2	1/6
2	658,29	1,16	25,1	63,6	1/6
3	628,67	1,2	25,1	24,4	1/10
3	623,67	1,26	24,5	35,5	1/10
			Jar-Test 6		
1	657,97	1,02	25,6	22,4	1/10
1	657,57	0,95	23,7	24	1/10
2	649,94	1,17	24,6	23,1	1/10
2	633,65	1,18	24,3	23,8	1/10
3	628,89	1,27	25,6	23,4	1/10
3	613,28	1,27	24,5	24,2	1/10

Material dilution 1		Sulphuric acid dose 1 g S°/L		Stirring time 6	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 2		
1	798,73	1,07	25,8	90,2	1/6
2	780,56	1,04	24,9	93,2	1/6
4	731,38	1,18	24,7	98,5	1/6
5	727,79	1,22	24,8	101,3	1/6
5	790,04	1,21	25,1	316	
6	694,34	1,33	25,3	326	
			Jar-Test 3		
1	821,35	1	24,7	108,5	1/6
1	762,89	0,99	25,3	120,3	1/6
2	759,56	1,17	24,6	110,4	1/6
2	733,73	1,11	24,8	90,8	1/6
3	728,25	1,17	25,2	99,9	1/6
5	670,58	1,3	25,6	53,3	1/10
5	666,68	1,26	25,8	52,7	1/10
6	649,2	1,34	25,4	56,9	1/10
6	646,13	1,4	24,9	60,1	1/10
			Jar-Test 5		
1	681,94	0,9	26,2	212	
1	769,58	0,99	26,1	101,2	1/6
1	763,59	1,02	25,6	86,9	1/6
2	758,56	1,06	25,4	59,9	1/10
2	743,62	1,05	25	67,1	1/10
3	739,62	1,09	25,5	65,2	1/10
5	688,93	1,26	24,4	74,1	1/10
5	683,35	1,23	25,1	68,9	1/10
6	671,04	1,33	24,6	70,1	1/10
6	666,64	1,36	24,8	69,6	1/10
Material dilution 1/2		**Sulphuric acid dose 1 g S°/L**		**Stirring time 6**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 2		
1	720,18	1,17	25,2	71,4	1/6
2	702,1	1,19	24,3	68,6	1/6
4	649,78	1,28	24,5	72	1/6
5	645,98	1,36	24,2	77,9	1/6
5	629,14	1,37	24,8	227	
6	607,74	1,43	25	235	
			Jar-Test 3		
1	660,31	1,16	24,5	219	
1	699,34	1,17	25,1	302	
2	696,54	1,16	24,8	284	

2	677,8	1,21	24,2	271	
3	673,74	1,22	24,4	307	
5	598,44	1,35	24,7	216	
5	595,46	1,32	25,1	215	
6	573,03	1,4	25,6	210	
6	570,02	1,49	25,2	199,2	
			Jar-Test 5		
1	606,03	1,17	25,7	101,4	
1	698,68	1,14	25,5	61,4	1/6
1	693,21	1,17	25,1	59,6	1/6
2	688,04	1,22	25,1	35,3	1/10
2	671,46	1,24	24,6	37,5	1/10
3	667,13	1,31	25,2	38,7	1/10
5	599,07	1,43	23,8	45,4	1/10
5	593,67	1,42	24,7	40,4	1/10
6	571,12	1,46	23,9	44	1/10
6	562,72	1,49	24,3	44,4	1/10
Material dilution 1/10		**Sulphuric acid dose 1 g S°/L**		**Stirring time 6**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 2		
1	665,3	1,43	25,1	17,86	1/6
2	650,88	1,46	24,2	26,6	1/6
4	600,26	1,59	24,2	31,1	1/6
5	596,14	1,65	24,1	80,1	
5	576,3	1,63	24,5	82,1	
6	555,14	1,73	24,7	82,9	
			Jar-Test 3		
1	607,43	1,22	24,2	75,6	
1	634,32	1,31	25	64,8	
2	632,43	1,41	24,4	63,7	
2	613,14	1,36	24	69,6	
3	609,48	1,47	24,1	73,8	
5	526,13	1,56	24,5	49,3	
5	521,85	1,56	24,8	49,5	
6	495,14	1,58	25,3	49,1	
6	491,27	1,64	25,1	51,3	
			Jar-Test 5		
1	528,02	1,39	25,2	58,9	
1	634,43	1,42	25,3	79,2	
1	631,61	1,41	24,5	79,4	
2	628,15	1,55	24,8	76,8	
2	609,59	1,52	24,3	83,9	
3	606,55	1,69	25	82,6	

5	531,7	1,73	23,7	65,2	
5	527,06	1,74	24,2	66,5	
6	502,57	1,8	23,7	63,7	
6	497,4	1,8	23,8	64,6	
Material dilution 1		**Sulphuric acid dose 3 g S°/L**		**Stirring time 6**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
		Jar-Test 2			
1	1093,69	0,77	26,2	107,8	1/10
2	1076,11	0,86	25,5	119,4	1/10
4	1027,75	0,97	25,3	119,4	1/10
5	1024,03	1,05	25,3	121,8	1/10
5	1010,49	1,08	25,4	124,9	1/10
6	995,56	1,19	25,7	156,7	1/6
		Jar-Test 3			
1	1041,97	0,77	25,3	147,1	1/6
1	1073,09	0,83	25,8	217	1/4
2	1069,51	0,98	26	208	1/4
2	1052,08	0,93	25	224	1/4
3	1047,45	0,99	25,3	216	1/4
5	996,67	1,06	25,7	130,1	1/10
5	992,87	1,11	25,9	117,6	1/10
6	978,71	1,16	26,2	120,1	1/10
6	977,24	1,21	26,3	127,5	1/10
		Jar-Test 5			
1	1012,07	0,77	26,6	144,1	1/6
1	1075,2	0,77	26,3	153	1/6
1	1071,63	0,8	25,9	161,4	1/6
2	1066,27	0,86	25,7	126,8	1/10
2	1053,15	0,91	25,5	133,5	1/10
3	1049,45	0,96	25,7	136,9	1/10
5	1005,7	1,09	24,8	145,7	1/10
5	1000,73	1,11	25,6	131,5	1/10
6	989,92	1,22	25,2	132,4	1/10
6	985,41	1,21	25,3	136,7	1/10
Material dilution 1/2		**Sulphuric acid dose 3 g S°/L**		**Stirring time 6**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
		Jar-Test 2			
1	861,7	0,92	26	69,4	1/10
2	846,68	0,9	25,2	81,4	1/10
4	808,16	1,07	24,9	79,4	1/10
5	804,4	1,17	25,1	61,4	1/10
5	790,04	1,16	25,2	77,4	1/10
6	774,25	1,28	25,5	116,6	1/6

			Jar-Test 3		
1	821,4	0,97	24,9	123,1	1/6
1	838,75	0,92	25,6	165,3	1/6
2	835,3	1,01	25,7	156,1	1/6
2	817,93	1	24,7	149,7	1/6
3	813,49	1,12	25,1	164,8	1/6
5	759,69	1,27	25,5	63	1/10
5	755,48	1,27	25,8	67,6	1/10
6	739,78	1,32	25,9	63,2	1/10
6	738,2	1,35	25,8	61,7	1/10
			Jar-Test 5		
1	773	0,86	26,5	105	1/6
1	839,08	0,93	26,1	114,7	1/6
1	835,51	0,86	25,5	119,7	1/6
2	829,65	0,98	25,6	81,3	1/10
2	815,76	0,98	25,5	86,7	1/10
3	811,81	1,04	25,6	88,2	1/10
5	766,5	1,15	24,8	96,5	1/10
5	761,16	1,15	25,3	88,2	1/10
6	748,09	1,25	24,8	89,2	1/10
6	745,61	1,27	25,1	91,4	1/10
Material dilution 1/10		**Sulphuric acid dose 3 g S°/L**		**Stirring time 6**	
Day	**Weight (g)**	**pH**	**Temperature (°C)**	**Conductivity (sm)**	**Dilution**
			Jar-Test 2		
1	687,78	1,07	25,5	48,7	1/6
2	672,61	1,05	24,6	51,7	1/6
4	612,81	1,18	24,6	57	1/6
5	608,75	1,32	24,5	184,4	
5	594,2	1,31	25,1	189,4	
6	575,25	1,4	25,5	172	
			Jar-Test 3		
1	625,61	1,17	24,5	165,2	
1	672,77	1,17	25,2	232	
2	670,87	1,25	25,1	226	
2	655,58	1,27	24,3	235	
3	651,33	1,33	24,4	241	
5	598,51	1,39	25	188,7	
5	595,19	1,37	25,3	188,5	
6	580,4	1,4	25,9	186	
6	577,96	1,44	25,3	187,6	
			Jar-Test 5		
1	613,7	1	26,2	176,8	
1	668,74	1,08	26	68,4	1/6

1	664,83	1,09	25,2	65,4	1/6
2	658,61	1,13	25,4	24,7	1/10
2	644,26	1,18	25,4	26,6	1/10
3	639,69	1,24	25,3	26,5	1/10
5	596	1,34	24,6	30,3	1/10
5	586,76	1,36	25	29,5	1/10
6	570,23	1,4	24,3	28,6	1/10
6	564,59	1,45	24,7	29,6	1/10

Annex 6.ofcumi Clarified and settled characterization

	Jar-Test 1		
	Clarified		
Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,21	569	30
2	1,42	290	15
3	1,79	75	7,5
4	1,04	1015	20
5	1,22	655,5	30
6	1,4	142,4	7,5
	Settled		
1	1,22	421,5	
2	1,38	187,8	
3	1,76	74,2	
4	1,08	674	
5	1,22	643	
6	1,41	145	
	Jar-Test 2		
	Clarified		
Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,36	798,6	2,5
2	1,54	351	7,5
3	1,75	68,8	2,5
4	1,23	1404	2,5
5	1,49	1123,2	5
6	1,62	283	5
	Settled		
1	1,38	958,8	
2	1,54	358	
3	1,73	168,5	
4	1,21	138	
5	1,54	1099,8	
6	1,65	264	
	Jar-Test 3		
	Clarified		
Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,32	511,2	20
2	1,56	336,8	10
3	1,91	88,4	5
4	1,18	1032	2,5
5	1,42	832,8	5

6	1,6	160,8	5
		Settled	
1	1,31	411,6	
2	1,57	320,8	
3	1,93	133,6	
4	1,21	884	
5	1,47	654	
6	1,63	206	
		Jar-Test 4	
		Clarified	
Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,24	543	10
2	1,42	321	25
3	1,71	58,5	2,5
4	1,16	1066	30
5	1,27	709	25
6	1,46	145,5	2,5
		Settled	
1	1,26	526	
2	1,4	292	
3	1,67	61,1	
4	1,14	899	
5	1,28	676	
6	1,48	187,7	
		Jar-Test 5	
		Clarified	
Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,29	756	7,5
2	1,53	387	5
3	1,84	69,5	2,5
4	1,14	1462	2,5
5	1,22	1013	5
6	1,59	175	2,5
		Settled	
1	1,28	687	
2	1,63	482	
3	1,84	68,2	
4	1,11	1273	
5	1,18	909	
6	1,56	312	
		Jar-Test 6	
		Clarified	

Jar	pH	Conductivity (Sm)	Cu (mg Cu/L)
1	1,25	710	7,5
2	1,44	331	10
3	1,72	68,2	2,5
4	0,95	1383	10
5	1,06	846	15
6	1,29	164	5
	Settled		
1	1,21	651	
2	1,54	397	
3	1,69	65,7	
4	0,98	1236	
5	1,08	898	
6	1,31	268	